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INTRANASAL AND AEROSOL EXPOSURE OF GERBILS TO 'MYCOPLASMA PNEUM--ETC(U)
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INTRANASAL AND AEROSOL EXPOSURE OF GERBILS

TO MYCOPLASMA PNEUMONIAE[1,2,3]

JOSEPH V. JEMSKI, PhD, AND SAMUEL V. MACHOTKA, DVM

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INTRANASAL AND AEROSOL EXPOSURE OF GERBILS TO MYCOPLASMA PNEUMONIAE

Running title: GERBIL RESPONSE TO MYCOPLASMA PNEUMONIAE

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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INTRANASAL AND AEROSOL EXPOSURE OF GERBILS TO *MYCOPLASMA PNEUMONIAE*[1,2,3]

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micrometers

SUMMARY. *Mycoplasma pneumoniae* was administered to Mongolian gerbils by intranasal instillation or by exposure to small-particle aerosols (2 μm). From 7 - 14 days after exposure, approximately 5 $\log_{10}$ of mycoplasma were recovered from the lungs of the majority of exposed gerbils. Lung lesions were minimal in the infected animals and not distinguishable from those of control animals administered sterile broth medium. Rechallenge of gerbils 30 days after an initial infection did not result in an increase of pulmonary pathology over that of control animals. Serum cholesterol levels and peripheral blood values were not altered in either initially infected or reinfected gerbils.

FOOTNOTES

[1] From the U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

[2] In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

[3] The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

Information is not available on the response of the Mongolian gerbil (*Merionus unguinculatus*) to experimental infection with *Mycoplasma pneumoniae*, an important pathogen of the human respiratory tract. The gerbil is a lipemic animal with serum cholesterol concentrations often exceeding 110 mg/dl (1). Similar cholesterol levels have been recorded for the hamster (2), an animal widely used for studying *M. pneumoniae* infections. The hamster, although responding to *M. pneumoniae* infections in a temporal sequence similar to man (3) does not die nor demonstrate overt clinical signs even in the presence of severe histopathological changes in the respiratory tract (4). Diagnosis of experimentally induced mycoplasma infection and the severity of the disease in the hamster is based on isolation of the organisms from the respiratory tract and the histological appearance of the pulmonary lesions. Since both the hamster and gerbil characteristically possess similarly high levels of serum cholesterol, the use of the gerbil as a potential alternate model for experimental *M. pneumoniae* infections was evaluated. In addition, since cholesterol is an essential metabolite for *M. pneumoniae* (5), it was of interest to determine the effect of the infection on serum cholesterol levels of the gerbil. This paper, therefore, describes the response of the gerbil exposed to virulent *M. pneumoniae* administered to the respiratory tract either by the intranasal route or as a small-particle aerosol.

MATERIALS AND METHODS

Experimental animals. Outbred, adult, male Mongolian gerbils (*Merionus unguinculatus*) Tum (Mon)[1], weighing 60-75 g were used in all experiments. Animals were quarantined at least one week prior to use. Cultures from gerbils prior to experimentation indicated that their lungs were free of indigenous mycoplasma, and sera were negative for complement-fixing antibody against *M. pneumoniae*. After infection or challenge, animals were husbanded 4-6 per cage and given water and commercial feed pellets[2] ad libitum. Cross-infection among experimental groups was avoided by maintaining each group in separate ventilated biologic safety cabinets. The temperature in the cabinets was 23-25°C, and the relative humidity approximately 42%.

M. pneumoniae culture. A third passage culture of *M. pneumoniae* strain PI 1428[3] was grown in Hayflick's medium (6) and used for initial infection and subsequent challenge of the gerbils.

Intranasal inoculation of gerbils. Gerbils were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital[4] (0.03 mg/g body weight). A volume of 150 µl containing approximately 10^6 colony forming units (CFU) of *M. pneumoniae* in complete mycoplasma broth (6) was instilled in the anterior nares by means of an Oxford micropipette[5]. Control animals similarly anesthetized received 150 µl of sterile, unseeded mycoplasma broth by the intranasal (i.n.) route. All i.n. inoculations were performed in a laminar air flow hood.

FOOTNOTES

[1] Tumblebrook Farms, West Brookfield, MA.

[2] Laboratory Chow[R], Ralston Purina, Inc., St. Louis, MO.

[3] Originally obtained from Dr. R. Chanock, National Institutes of Health, Bethesda, MD.

[4] Veterinary Nembutal, Abbot Laboratories, N. Chicago, IL.

[5] Oxford Laboratories, Foster City, CA.

Aerosol exposure of gerbils. Unanesthetized gerbils were exposed to an aerosol of a broth suspension of *M. pneumoniae* for 20 minutes in a modified Henderson aerosol apparatus (7) equipped with a Collison spray device (8). Based on earlier calibration studies, the aerosol particles had a mass median diameter of 2 µm. The mycoplasma concentration of the aerosol was estimated from aerosol samples collected in all-glass impingers (9) containing 20 ml of mycoplasma broth. Presented mycoplasma aerosol dosages were computed using the following formula: colony forming units (CFU)/liter of aerosol x minute respiratory volume of the gerbil (0.05/liter/min) x duration of animal exposure in minutes (9).

Assay procedure. On day 7 or 14 after initial infection and at 14 days after rechallenge, gerbils to be assayed were injected i.p. with a lethal dose of pentobarbital. The vena cava was incised, and the blood collecting in the thoracic cavity was pipetted into a tube containing heparin. Total leukocyte concentrations were obtained on the heparinized blood with a Coulter counter.[6] Packed red-cell volumes were determined by a microhematocrit method[7], differential leukocyte estimates were obtained by manual counting of Wright-stained blood smears. Plasma cholesterol concentration was determined by automatic analysis[8]. Standard microtiter procedures were utilized to measure complement-fixing antibodies in plasma samples. Four units of M. pneumoniae antigen[9] were employed in these assays.

FOOTNOTES

[6] Coulter Electronics, Inc., Hialeah, FL.

[7] Autocrit[R], Clay-Adams, Parsippany, NJ.

[8] Auto Analyzer II, Technicon Corp., Tarrytown, NY.

[9] Obtained from Microbiological Associates, Bethesda, MD.

Microbiological and histological methods. In determining mycoplasma concentrations, the lungs were aseptically excised from anesthetized gerbils. Individual lungs were homogenized in a mortar and pestle, and sufficient complete mycoplasma broth was added to yield a 10% suspension (w/v). One-tenth ml of 10-fold serial dilutions of the lung suspension was inoculated on duplicate mycoplasma agar plates[10]. Inoculated plates were incubated for 3 weeks at 37°C, at which time they were examined and colonies enumerated.

Material from the anterior nares of gerbils was also cultured for mycoplasma. Small cotton-tipped swabs, moistened with sterile mycoplasma broth, were inserted first in one nostril, twisted, and then inserted in the second nostril. The swabs were streaked on the surface of duplicate mycoplasma agar plates which were examined for typical colonies after 3 weeks incubation at 37°C.

Lungs were prepared for histological examination by injecting 3 ml of 10% buffered formalin through the right ventricle of the heart to flush residual blood out of pulmonary vessels. The trachea was then ligated and the lung perfused with formalin via tracheal cannulation using a 22 gauge needle. The ligated trachea and perfused lung were carefully excised and placed in formalin fixative. Entire lobes were sectioned and stained with hematoxylin and eosin and examined for peribronchial cellular infiltration and endobronchial exudate indicative of *M. pneumoniae* lesions.

FOOTNOTES

[10] PPLO agar, obtained from Microbiological Associates, Bethesda, MD.

Body and organ weights. Gerbils were weighed at the onset of experiments and on selected days thereafter. Liver, spleen and kidney were excised, trimmed of extraneous tissue and weighed on an electronic digital reading balance.[11] Sections of these organs also were fixed in formalin for histological examination.

FOOTNOTES

11 Digimetric Balance, Sybron Corp., Englewood, CO.

RESULTS

M. pneumoniae, at $\log_{10}$ concentrations of 5.7 - 6.2 CFU, were administered to gerbils either by i.n. instillation or as small-particle aerosols (SPA). The frequency of infection and the degree of lung pathology in these animals were determined 7 and 14 days after exposure (Table 1). M. pneumoniae was isolated from the lungs of more than 50% of the i.n. infected gerbils at 7 and 14 days and from the aerosol-exposed group at 14 days. Consistently similar concentrations of mycoplasma were measured in the lungs of gerbils at both assay periods for all infected groups regardless of the method used to infect the animals.

Although approximately 5 $\log_{10}$ of mycoplasma were isolated from the lungs, respiratory distress was not clinically observed, nor were lesions histologically attributable to M. pneumoniae demonstrable in lungs from any of the animals. The minimal to mild lesions observed in both the control and infected gerbils consisted of a subacute peribronchiolitis, suggestive of indigenous lesions seen in murine chronic respiratory disease.

M. pneumoniae was isolated from about 25% of the nasal swabs taken at 7 and 14 days from the gerbils infected intranasally. This indicates that mycoplasma colonized the upper respiratory tract of these animals for at least two weeks. No mycoplasma were isolated from the nares of the gerbils exposed to the SP aerosols.

Only 2 of the 69 exposed animals developed serum complement-fixing (CF) antibody titers; their serum titers were low. Similar minimal CF titers have been reported for hamsters infected by the i.n. route or by SPA with M. pneumoniae (4).

Gerbils exposed previously to M. pneumoniae by intranasal instillation or SPA were challenged 30-36 days later to determine whether they were immune to rechallenge (Table 2). Control gerbils which had initially received sterile broth medium intranasally were challenged also. The challenge dose for all 3 groups was 5.5 - 6 $\log_{10}$ CFU/animal and was administered either intranasally or as SPA. All animals were examined for infection 14 days after the challenge.

The infection rates in the broth controls were 61% and 86% for the i.n. and SPA challenged gerbils, respectively, a difference that was not statistically significant ($P \leq 0.05$ by a two-tailed Fisher's exact test). These results agreed with the 14-day data observed after primary infection (Table 1). SPA rechallenge of previously infected animals resulted in an infection rate of about 61%, regardless of initial route of infection.

In contrast, the infection rate (25%) of the animals previously infected by i.n. instillation and then rechallenged by the same route was significantly lower than all other challenged groups ($P \leq 0.0.$, by Fisher's exact test).

The concentration of mycoplasma in the lungs of reinfected gerbils was not significantly different from the mycoplasma concentrations estimated for the broth control group challenged with SPA ($P \leq 0.01$, Test of Least Significant Differences following Analysis of Variance). The similarity of these concentrations indicated that prior infection

apparently did not confer significant resistance to subsequent growth in the lungs. As in gerbils infected only once, the reinfected animals showed no overt signs of illness nor significant pulmonary histopathology.

Blood cholesterol levels are shown in Table 3. Regardless of method or number of infections the serum cholesterol values of all the gerbils were similar to those in the noninfected controls. These serum cholesterol levels also were in agreement with the 112 mg/dl values previously reported for normal gerbils (1).

Peripheral blood values, body and organ weights of infected and noninfected gerbils are presented in Table 4. Hematocrit values were in the normal range for both infected and control animals. Similarly, total and differential leukocyte counts were not altered by mycoplasma infection and were consistent with normal ranges reported in the literature (11).

The weights of liver, spleen and kidney were measured to determine the extent of infection-related changes. Compared to noninfected controls, no differences were seen. Histological examinations of these organs also indicated no abnormalities.

DISCUSSION

This study describes the response of the Mongolian gerbil to virulent M. pneumoniae administered to the respiratory tract either in small-particle aerosols or by intranasal instillation. In common with the hamster, the animal most utilized for research on M. pneumoniae infection, the infected gerbil did not exhibit discernible clinical illness. Histological examination of the respiratory tract of infected gerbils revealed no definitive lesions despite the presence of approximately 10^5 CFU of mycoplasma in the lungs 2 weeks after infection. This observation is consistent with that seen in the hamster in which significant progressive lung lesions were not always produced even in lungs which contained 10^6 CFU of mycoplasma (3).

The absence of clinical response may be due to the age of the gerbils; younger animals (less than 40 g) may be more susceptible to M. pneumoniae since greater susceptibility has been shown for young gerbils with other microorganisms. For example, Winsser reported that all young gerbils infected intranasally with Bordetella bronchiseptica developed pneumonia and died within a week, while older animals were more resistant and many survived; some survivors became carriers (12).

Evidence of some protection against pulmonary reinfection in gerbils previously infected with M. pneumoniae was seen only in the animals initially infected i.n. and then reinfected by the same route. A possible explanation for this protection is that the inoculum remains in closer contact with local antibody (presumably IgA) producing cells (13) than it does in gerbils infected with small-particle

aerosols. Small particle aerosols are deposited predominantly deep in the lung parenchyma (10) where immunoglobulin-containing cells are not present (14).

Our studies also afforded the opportunity to obtain some physiological data on the gerbil, an animal not currently widely used in infectious disease research. We did confirm the high serum cholesterol levels previously reported for the Mongolian gerbil (1). We also demonstrated that serum cholesterol levels were not affected by infection with a strain of mycoplasma that required cholesterol as a metabolite, probably because M. pneumoniae remains localized in the respiratory tract. Other physiological parameters not changed by mycoplasma infection were hematocrit levels, total and differential leukocyte counts and weights of liver, spleen and kidney.

The apparent resistance of the gerbil to respiratory infection with virulent M. pneumoniae indicates that these animals also would be resistant to natural respiratory infection with murine mycoplasmas. This, then, suggests an applicability of the gerbil as a suitable murine model for infectious disease research.

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TABLE 1

Response of gerbils to *M. pneumoniae* administered intranasally (i.n.) or by aerosol (SPA)

Inoculum (Dose, $\log_{10}$ CFU $\pm$ SD)	Time After Inoc (days)	No. positive/no. tested: Lung Infection	Lung Count[a] ($\log_{10}$ CFU $\pm$ SD)	Nasal Swab	Serum CF[b] (titer)
i.n. (150 µl)					
Sterile broth (0)	7	0/13	0	0/6	0/20
	14	0/15	0	0/8	0/16
M. pneumoniae (6.2 $\pm$ 0.55)	7	10/19	4.96 $\pm$ 1.64	3/12	0/27
	14	12/21	4.65 $\pm$ 2.10	3/14	1/26 (4)
SPA					
M. pneumoniae (5.7 $\pm$ 0.08)	7	2/16	4.72 $\pm$ 1.75	0/6	0/6
	14	6/10	4.88 $\pm$ 1.20	0/10	1/10 (8)

[a] Geometric mean titer of colony forming units (CFU) per lung for positive animals.

[b] Number in parenthesis is reciprocal of CF titer.

TABLE 2

Response of gerbils to rechallenge with *M. pneumoniae*

Initial Challenge	Method of Rechallenge	Rechallenge[a] ($\log_{10}$ CFU $\pm$ SD)	Lung Infection, No. pos./No. tested	Lung Count[b] ($\log_{10}$ CFU $\pm$ SD)
Broth controls (i.n.)	i.n.	6.08 $\pm$ 0.55	11/18	6.57 $\pm$ 0.96
	PSA	5.53 $\pm$ 0.33	6/7	5.17 $\pm$ 1.04
M. pneumoniae (i.n.)	i.n.	6.08 $\pm$ 0.55	7/28[c]	4.69 $\pm$ 0.80
	SPA	5.53 $\pm$ 0.33	6/10	4.49 $\pm$ 0.88
M. pneumoniae	SPA	5.53 $\pm$ 0.33	8/13	4.23 $\pm$ 0.94

[a] Animals rechallenged 30-36 days after the initial infection assayed 14 days after rechallenge.

[b] Geometric mean, $\pm$ SD for positive animals only.

[c] Significantly different from all other groups; $P \leq 0.01$ (Fisher's exact test, 2-tail).

TABLE 3

Cholesterol values of noninfected and infected gerbils

Treatment	No. Gerbils	Body weight (gm)	Cholesterol (mg/dl) Mean $\pm$ SD
Broth controls (noninfected)	10	67 (63-69)[c]	107.7 $\pm$ 26.7
M. pneumoniae[a] (initial infection)	42	67 (58-74)	112.7 $\pm$ 29.1
M. pneumoniae[b] (reinfected)	25	66 (60-74)	108.7 $\pm$ 27.8

[a] Animals assayed 7-14 days after infection.

[b] Animals assayed 14 days after reinfection.

[c] Mean (range).

TABLE 4

Peripheral blood values, body and organ weights of control and infected gerbils

Variables	No. gerbils	Broth controls (Mean ± SD)	No. gerbils	Initially infected (Mean ± SD)	No. gerbils	[illegible]infected (Mean ± SD)
Hematocrit (%)	40	38.5 ± 1.6	69	39.6 ± 2.7	36	37.8 ± 1.2
Total WBC (10^3)	30	8.5 ± 1.3	57	8.5 ± 1.9	36	7.0 ± 4.2
Lymphocytes (%)	34	79.0 ± 7.2	69	81.0 ± 6.5	36	78.0 ± 5.8
Neutrophils (%)	34	18.0 ± 7.1	69	16.0 ± 7.8	36	20.0 ± 6.5
Others (%)[a]	34	3.0 ± 1.8	69	3.0 ± 1.9	36	2.0 ± 1.2
Body weight (g)	51	67.0 ± 4.8	57	69.0 ± 6.5	40	73.0 ± 6.5
Liver (g)	30	2.83 ± 0.4	31	2.78 ± 0.4	22	2.78 ± 0.3
Spleen (g)	30	0.09 ± 0.01	31	0.09 ± 0.02	22	0.08 ± 0.02
Kidney (g)	25	0.28 ± 0.04	31	0.26 ± 0.05		

[a] Primarily monocytes; occasional basophils or eosiniphils, immature neutrophils.

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Mycoplasma pneumoniae was administered to Mongolian gerbils by intranasal instillation or by exposure to small-particle aerosols (2 μm). From 7 - 14 days after exposure, approximately 5 $\log_{10}$ of mycoplasma were recovered from the lungs of the majority of exposed gerbils. Lung lesions were minimal in the infected animals and not distinguishable from those of control animals administered sterile broth medium. Rechallenge of gerbils 30 days after an initial infection did not result in an increase of pulmonary pathology over that of control animals. Serum cholesterol levels and peripheral blood values

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